

Molecular cloning, expression, and immunolocalization of protein disulfide isomerase in excretory–secretory products from *Clonorchis sinensis*

Yue Hu · Lisi Huang · Yan Huang · Lei He ·
Fan Zhang · Wenfang Li · Pei Liang · Ran Li ·
Jiufeng Sun · Xiaoyun Wang · Chi Liang · Xuerong Li ·
Xinbing Yu

Received: 24 February 2012 / Accepted: 10 April 2012 / Published online: 27 April 2012
© Springer-Verlag 2012

Abstract Protein disulfide isomerase (PDI) is an essential catalyst of the endoplasmic reticulum with folding and chaperone activities in different biological systems. Here, PDI of *Clonorchis sinensis* (CsPDI) was isolated from the cDNA library of adult *C. sinensis*. The open reading frame contains 1,317 bp encoding 438 amino acids and shares 53 %, 49 %, and 43 % identity with PDI from *Bos taurus*, *Homo sapiens*, and *Schistosoma mansoni*, respectively. Two catalytic thioredoxin motifs CxxC were found in this sequence, which were characteristic domains of thioredoxin superfamily. The CsPDI protein was expressed and purified from *Escherichia coli* BL21 (DE3). According to western blotting analysis, the recombinant CsPDI could be recognized by anti-CsPDI rat serum, anti-excretory/secretory products rat serum, and serum of rat infected with *C. sinensis*, respectively. Quantitative real-time polymerase chain reaction showed that transcription level of CsPDI in the metacercaria stage was six and four times higher than that

in the adult worm and egg stage, respectively. Immunolocalization analysis showed CsPDI could be detected in the intestine, vitellarium, and intrauterine eggs of adult worm, as well as in the cyst wall and vitellarium of metacercaria. In addition, the strong fluorescence signal was observed both on the wall of bile duct and in the lumen of liver tissue of *C. sinensis*-infected cat. Those results demonstrated that CsPDI was a component of *C. sinensis* excretory–secretory products. The present study will enhance our understanding of biological functions of CsPDI and pave the way for further studies on host–parasite interaction during *C. sinensis* infection.

Introduction

Clonorchiasis is an important food-borne parasitic disease caused by *Clonorchis sinensis* infection. Thirty-five million people have been infected globally, of whom approximately 15 million are in China (Lun et al. 2005). Opportunistic infection of human being occurs by ingestion of raw or improperly cooked freshwater fish containing live encysted metacercariae (Sripa et al. 2010). The dwelling of *C. sinensis* adult worm in bile ducts results in a series of pathological changes such as pyogenic cholangitis, cholelithiasis, cholecystitis and hepatic fibrosis, even cholangiocarcinoma (Fried and Abruzzi 2010; Fried et al. 2011). Excretory–secretory products of *C. sinensis* (CsESPs) were considered to play important roles in the pathophysiologic responses (Pak et al. 2009) and parasite survival in the host bile ducts, where was a very hostile environment (Salazar-Calderon et al. 2003). Characterization of each component of ESPs and identification of accurate roles in the pathogenesis of *C. sinensis* infection may

Yue Hu and Lisi Huang contributed equally to this work.

Y. Hu · L. Huang · Y. Huang · L. He · F. Zhang · W. Li · P. Liang ·
R. Li · J. Sun · X. Wang · C. Liang · X. Li · X. Yu
Department of Parasitology, Zhongshan School of Medicine,
Sun Yat-sen University,
Guangzhou 510080, People's Republic of China

Y. Hu · L. Huang · Y. Huang · L. He · F. Zhang · W. Li · P. Liang ·
R. Li · J. Sun · X. Wang · C. Liang · X. Li · X. Yu (✉)
Key Laboratory for Tropical Diseases Control,
Ministry of Education, Sun Yat-sen University,
Guangzhou 510080, People's Republic of China
e-mail: yuhxteam@163.com

Y. Huang
School of Public Health, Sun Yat-sen University,
Guangzhou 510080, People's Republic of China

provide clues to find new strategies in prevention and therapy of clonorchiasis (Ma et al. 2007; Hu et al. 2009).

Protein disulfide isomerase (PDI) is an essential catalyst in different biological systems showing formation and reshuffling of disulfide bonds in the endoplasmic reticulum (ER) (Gilbert 1998). As molecular chaperones and foldases, PDI belongs to a member of thioredoxin superfamily to modulate the protein secretory pathway in eukaryotes (Freedman et al. 1994). The classical structure of PDIs involves two homologous catalytic domains, **a** and **a'**, comprising each an active site with a CxxC motif, separated by two homologous non-catalytic domains, **b**, **b'**, and a highly acidic C-terminal extension **c** (Byrne et al. 2009; Kozlov et al. 2010). Although PDIs were originally identified in the lumen of the ER, previous studies suggested that they also presented on the cell surface and modulated cellular interactions in a number of pathogens (Wilkinson and Gilbert 2004). It is well known that many major components of proteins related to adhesion and invasion of parasites are cysteine-rich and dependent on correct folding via disulfide bond formation of cysteines (Naguleswaran et al. 2005). In *Neospora caninum* and *Toxoplasma gondii*, both *NcPDI* and *TgPDI* were reported to play the important role in the parasite–host cell interaction (Liao et al. 2006; Robinson and Roy 2006). Moreover, PDI could also protect adult parasite against oxidative stress, which could be a relevant survival mechanism in *Fasciola hepatica* (Salazar-Calderon et al. 2003). Thus, PDIs have been considered to play significant roles in parasite–host cell interaction.

In the present study, we isolated a full-length cDNA of *CsPDI* from the adult *C. sinensis* cDNA library and did sequence analysis, cloning, expression, and immunolocalization of *CsPDI*, as well as its transcription levels in *C. sinensis* different stages.

Materials and methods

Sequence analysis of *CsPDI*

A full-length cDNA (clone number *Cs17f06*) encoding *CsPDI* was identified from a cDNA plasmid library of adult *C. sinensis* using bioinformatics tool provided by NCBI web site (<http://www.ncbi.nlm.nih.gov>). The physicochemical parameters, signal peptide, and structure characters of the deduced amino acid sequence were analyzed by using ExpPASy (<http://ca.expasy.org/tools>). Vector NTI suite 8.0 was used to analyze the homology of the target sequence.

Cloning, expression, and purification of the recombinant *CsPDI* (r*CsPDI*)

The polymerase chain reaction (PCR) was employed to amplify the target gene using sense primer 5'-CGCCGAATC

CAGGTTGAGGTGATG-3' (*EcoR* I, underlined) and anti-sense primer 5'-GCGCCTCGAGCTACAAGTCGCGTTT-3' (*Xho* I, underlined). PCR amplification condition was 94 °C for 1 min, 61 °C for 1 min, 72 °C for 1 min for 35 cycles, and then 72 °C for 10 min. The purified PCR products were purified and then digested by the corresponding restriction enzymes (*EcoR* I + *Xho* I), and cloned into the prokaryotic expression vector pET28a (+) (Novagen, USA). The recombinant plasmid was confirmed by DNA sequencing, and then transformed into *Escherichia coli* BL21/DE3 (Promega, USA). The protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37 °C for 4 h in Luria-Bertani medium (LB). The bacteria were harvested by centrifuging at 4 °C, and the recombinant protein was purified by His-Bind Resin Kit (Novagen, USA). The eluted protein was dialyzed in phosphate buffered saline (PBS, pH 7.4). The purity of the r*CsPDI* was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12 % gel). The final concentration of r*CsPDI* was detected by the BCA Assay Kit (Novagen, USA).

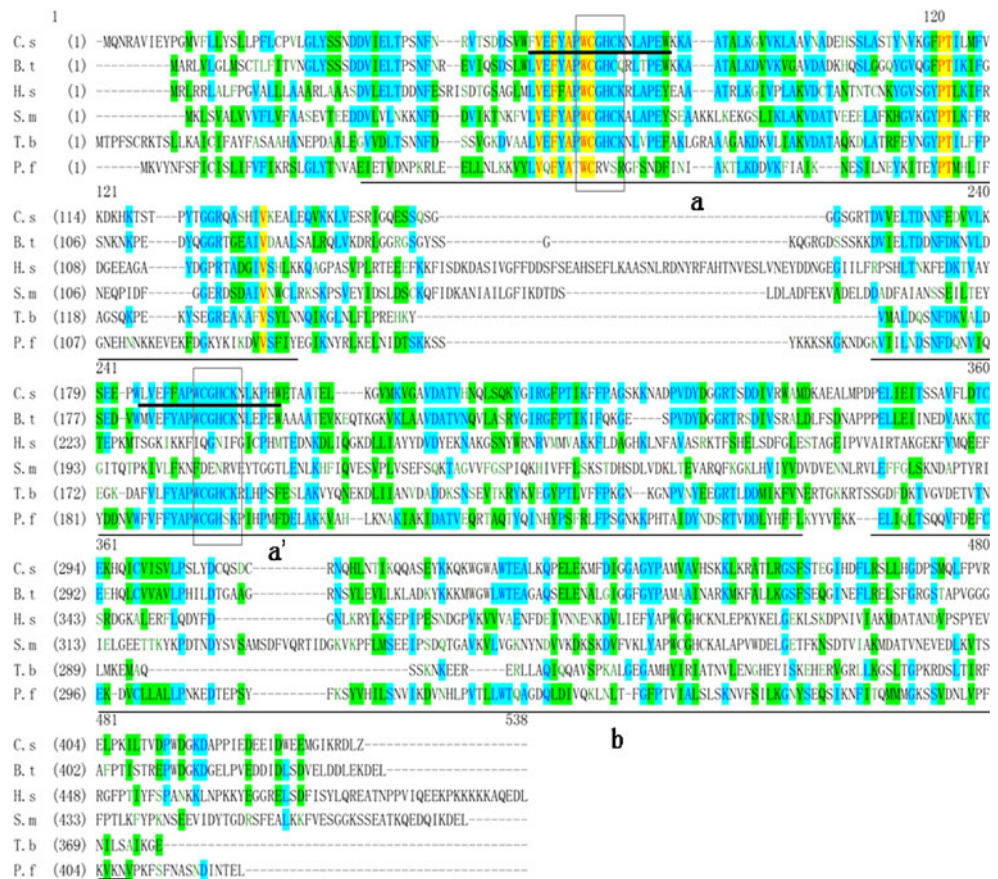
Preparation of *Cs*ESPs and antiserum

C. sinensis adult worms were isolated from the bile ducts of parasite-infected cats. The *Cs*ESPs were prepared as described previously (Hu et al. 2007) and stored at –80 °C. The purified r*CsPDI* (200 μg) and *Cs*ESPs (200 μg) were respectively emulsified with equal volume of complete Freund's adjuvant and subcutaneously injected into two 6-week-old male Sprague–Dawley (SD) rats. Meanwhile, the naive serum was taken before injection. Three booster injections were given with 100 μg purified protein containing incomplete Freund's adjuvant at 2 weeks interval. The antisera were collected 2 weeks after the last injection. The titers of the antisera were determined by enzyme-linked immunosorbent assay (ELISA).

Western blotting analysis

The purified r*CsPDI* and *Cs*ESPs were subjected to SDS-PAGE (12 %) and subsequently electrotransferred onto the polyvinylidene difluoride (PVDF) membrane (Whatman, USA) at 100 V for 1.5 h. The PVDF membrane was blocked with PBS (pH 7.4) containing 5 % skim milk for 2 h at room temperature, washed five times with PBS (pH 7.4), and then incubated with anti-*CsPDI* rat serum (1:100 dilutions), naive rat serum (1:100 dilutions), *C. sinensis*-infected rat serum (1:100 dilutions), His-Tag monoclonal antibody (mAb-His) (Novagen, USA, 1:2,000 dilutions), and anti-*Cs*ESPs rat serum (1:100 dilutions) for overnight at 4 °C, respectively. The membrane was incubated with horseradish peroxidase (HRP)-conjugated goat

Fig. 1 Amino acid sequence alignment of PDI from *Clonorchis sinensis* with that of PDI from other species. *C. sinensis* (*C. s.*), *Bos taurus* (*B. t.*, NP_001193274.1), *Homo sapiens* (*H. s.*, CAA89996.1), *Schistosoma mansoni* (*S. m.*, XP_002576968.1), *Trypanosoma brucei* (*T. b.*, XP_845754.1), and *Plasmodium falciparum* (*P. f.*, XP_001348023.1). The conserved site (aa_{55–73} and aa_{184–202}) of thioredoxin family was indicated with **bold line**, the redox sites WCGHCK (aa_{62–67} and aa_{191–196}) were indicated with **gray frame**, and the proposed delimitations of **a**, **a'** (catalytic domains) and **b** (non-catalytic domains) were showed by a **line**



anti-rat/anti-mouse IgG (ProteinTech Group, USA, 1:2,000 dilutions) for 2 h at room temperature after washing. The color was developed with diaminobenzidine (DAB) substrate solution.

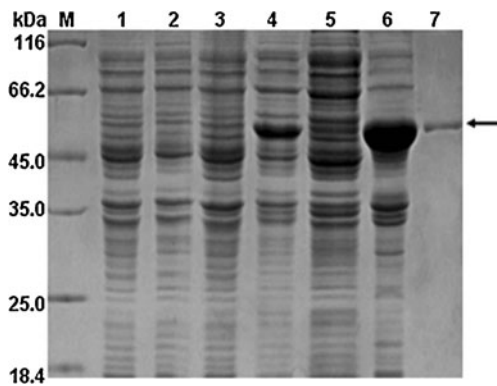


Fig. 2 SDS-PAGE analysis of expressed product of pET-28a (+)-*CsPDI* in *Escherichia coli* BL21/DE3. Protein molecular weight marker (*M*); pET-28a(+) transformants without IPTG induction (*lane 1*); pET-28a(+) transformants with IPTG induction (*lane 2*); pET-28a(+)-*CsPDI* transformants without IPTG induction (*lane 3*); pET-28a(+)-*CsPDI* transformants with IPTG induction (*lane 4*); supernatant of the lysate of pET-28a (+)-*CsPDI* transformants (*lane 5*); precipitation of the lysate of pET-28a (+)-*CsPDI* transformants (*lane 6*); the purified recombinant *CsPDI* protein (*lane 7*)

Quantitative real-time PCR analysis of *CsPDI* gene expression

The *C. sinensis* adult worms, eggs, and metacercariae were collected as previously described (Chen et al. 2011). Total RNA was extracted from them using TRIZOL (Invitrogen, USA) according to the manufacturer's instructions. The sense and antisense primers for *CsPDI* were 5'-TCAGTCC GATTGTGCGTAACCA-3', 5'-TCGTGAATACCC TCCGTGCTA-3', and primers of *Csβ-actin* (GenBank accession number EU109284.1), as internal control (Yoo et al. 2009), were 5'-ACCGTGAGAAGATGACGCAGA-3' and 5'-GCCAAGTCCAACGAAGAATT-3', respectively. The condition of real-time PCR was 95 °C for 10 min, and then 95 °C for 15 s, 60 °C for 10 s, and 72 °C for 60 s for 40 cycles; the reaction was finally ended up with a cooling step to 40 °C using SYBR Premix ExTaq™ II (Takara, Japan). The reaction was conducted in LightCycler™ system (LC480), and the data were analyzed according to the 2^{-ΔΔCt} method (Pfaffl 2001).

Immunohistochemical localization of *CsPDI*

The *C. sinensis* adult worm, metacercariae, and the liver tissue of *C. sinensis*-infected cat were fixed in 4 % paraformaldehyde,

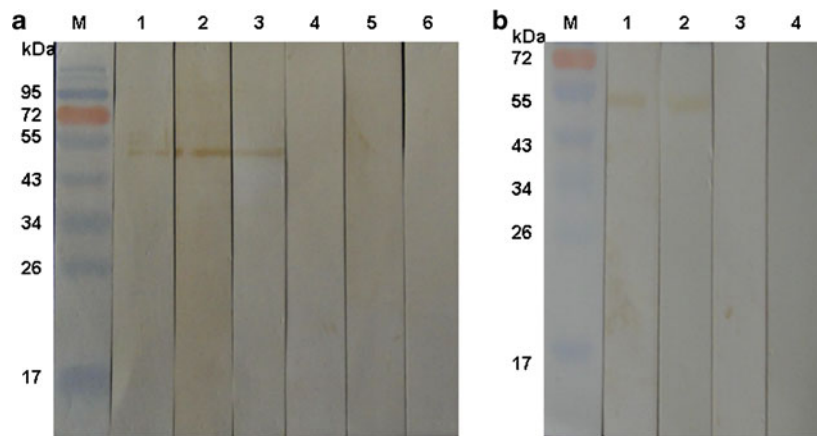


Fig. 3 Western blotting analysis of *CsPDI*. **a** Western blotting analysis of recombinant *CsPDI*. Purified r*CsPDI* was probed with mAb-His, anti-r*CsPDI* rat serum, and serum from *Clonorchis sinensis*-infected rat (lanes 1–3, respectively); no reaction was detected in the naive serum control (lanes 4–6). **b** Identification

of *CsPDI* as an excretory–secretory product. Anti-*CsPDI* serum was detected by the r*CsPDI* (lane 1); anti-r*CsPDI* serum was detected by ESPs (lane 2); no reaction was detected by the naive serum control (lanes 3–4)

embedded with paraffin, and sliced into sections of 4–5 μm in thickness. The sections were deparaffinized by dimethylbenzene three times and dehydrated by gradient alcohol. Then, the sections were blocked with normal goat serum for 2 h at room temperature, and incubated with anti-*CsPDI* rat serum (1:200 dilutions in 0.1 % BSA-PBS) and naive rat serum (1:200 dilutions) for overnight at 4 °C, respectively. The sections were incubated with CyTM 3-conjugated AffiniPure goat anti-rat IgG (1:400 dilutions) for 1 h at room temperature in darkness and quenched for 15 min, then imaged under fluorescence microscope (Carl Zeiss, Germany).

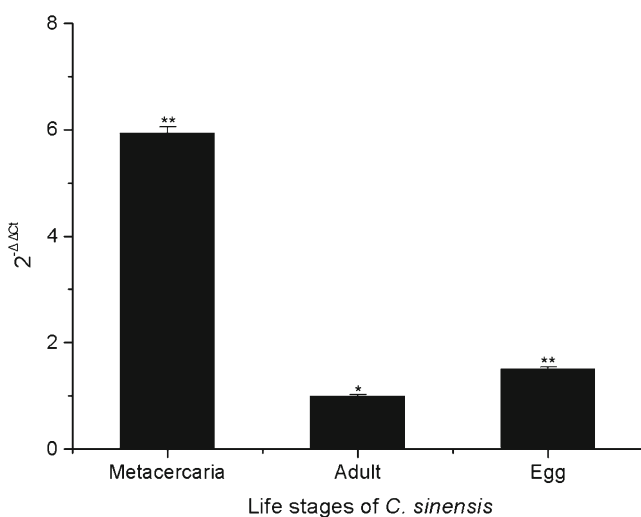


Fig. 4 Quantitative real-time PCR analysis of transcripts of *CsPDI* normalized by *Csβ-actin*. The data were analyzed by software package SPSS13.0. **p* value≤0.05; ***p* value≤0.01

Results

Sequence analysis of *CsPDI*

Open reading frame (ORF) of *CsPDI* was identified from the adult *C. sinensis* cDNA library. The ORF contained 1,317 bp encoding 438 amino acids. The predicted molecular mass (MW) of *CsPDI* was 52 kDa, and the isoelectric point (PI) was 6.57. No signal peptide or transmembrane domain was found. Two thioredoxin superfamily domains were found in the position of aa_{36–135} (**a** domain) and aa_{164–267} (**a'** domain). These domains comprised thioredoxin family conserved sites (aa_{55–73} and aa_{184–202}), which were homologous to bacterial thioredoxin and contained the putative redox site WCGHCK (Salazar-Calderon et al. 2003) (Fig. 1). BLASTx analysis showed that the deduced amino acid sequence was homologous with PDI of *Bos taurus*, *Homo sapiens*, *Schistosoma mansoni*, *Trypanosoma brucei*, and *Plasmodium falciparum* with 53 %, 49 %, 43 %, 36 %, and 25 % identities, respectively (Fig. 1).

Expression and purification of r*CsPDI*

The purified r*CsPDI* presented a single band at around 52 kDa, which coincided with the prediction of bioinformatics analysis (Fig. 2). The final concentration of recombinant protein was 200 $\mu\text{g}/\text{ml}$.

Western blotting analysis

The r*CsPDI* was recognized by mAb-His, anti-*CsPDI* rat serum, and *C. sinensis*-infected rat serum, while no corresponding band was detected by naive rat serum (Fig. 3a). The r*CsPDI*

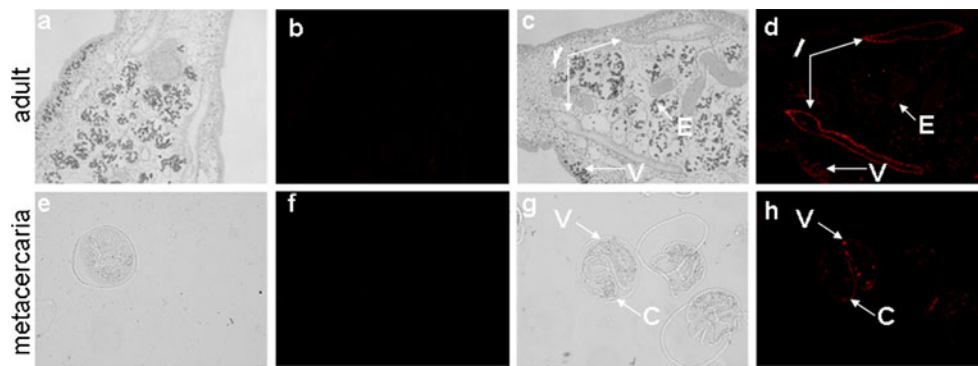


Fig. 5 Immunolocalization of CsPDI in the adult worm and metacercaria of *Clonorchis sinensis*. **a, c, e, g** Adult worm and metacercaria under optical microscope. **b, d, f, h** The same part under fluorescence microscope. **d, h** Probed with anti-CsPDI rat serum. **b, f** Probed with

naive serum as negative controls. The images were magnified at $\times 50$ for adult worm and $\times 400$ for metacercaria, respectively. *I* intestine, *V* vitellarium, *E* egg, *C* cyst wall

was probed by anti-CsESPs serum, and CsESPs was probed by anti-CsPDI serum, while no reaction was detected by naive serum control (Fig. 3b).

Transcript analysis of CsPDI at the development stages of *C. sinensis*

The transcripts of CsPDI were detected in the development stages of adult worm, metacercaria, and egg. Normalized by Cs β -actin, the transcription level of CsPDI in the metacercaria stage was six times higher than that in adult worm, whereas the expression level of CsPDI mRNA in the egg stage was one and a half times higher than that in adult worm (Fig. 4).

Immunohistochemical localization of CsPDI in the adult worm, metacercaria, and the liver tissue of *C. sinensis*-infected cat

CsPDI was detected in the sections of adult worm and metacercaria. Specific fluorescence was detected in the intestine, vitellarium, and intrauterine eggs of adult worm, as well as in the vitellarium and cyst wall of metacercaria. No specific fluorescence was observed in the adult worm and metacercaria when treated with naive serum (Fig. 5). In the liver tissue of *C. sinensis*-infected cat, specific fluorescence signal was observed on the wall of bile duct and in the lumen of liver tissue (Fig. 6).

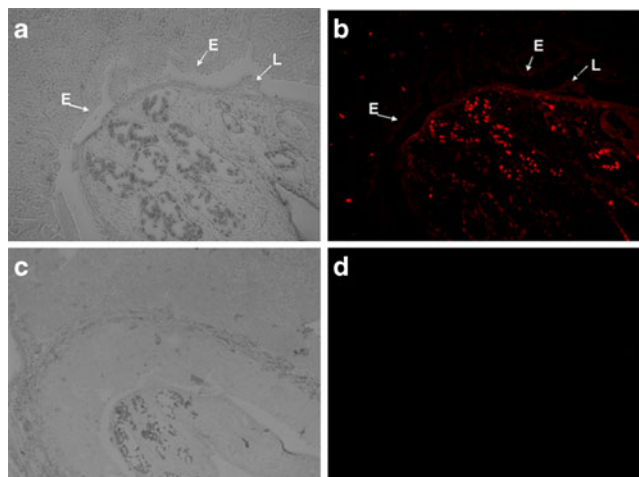


Fig. 6 Immunolocalization of CsPDI in the liver tissue of *Clonorchis sinensis*-infected cat. **a, c** Adult worm and its surrounding bile duct under optical microscope. **b, d** The same part under fluorescence microscope. **b** Probed with anti-CsPDI rat serum. **d** Probed with naive serum as a negative control. The images were magnified at $\times 50$. *E* wall of bile duct, *L* lumen of bile duct

Discussion

In this study, we isolated a full-length cDNA of CsPDI from cDNA plasmid library of adult *C. sinensis*. The protein showed high similarities with PDIs of other organisms. The CsPDI comprised two catalytic thioredoxin motifs CxxC, which were characteristic domains of thioredoxin superfamily. The purified rCsPDI with 6 \times His Tag was obtained in our studies. Analysis of quantitative real-time PCR indicated that CsPDI was expressed across all development stages of the parasite which occur in the definitive host, i.e., metacercaria, adult worm, and egg. It was also clarified in the immunolocalization assay. CsPDI was confirmed as a component of CsESPs by western blotting and immunohistochemical localization of the liver tissue of *C. sinensis*-infected cat in this study.

The members of the PDIs were expressed in the lumen of ER to participate in the folding and maturation of newly synthesized proteins (Hong and Soong 2008). The C-terminus sequence of PDI in mammal functioned as a

retention signal for luminal proteins in ER, which prevented them from being exported through the secretory pathway (Finken et al. 1994). However, the C-terminus sequence could be deleted from the polypeptide of PDIs without affecting any of its major functions (Freedman et al. 2002). In this study, we found that *CsPDI* contained a typical redox-active site **a** (aa_{58–67}, FYAPWCGHCK) as an N-terminal signal peptide (Rubotham et al. 2005), but not possessed the C-terminus sequence **c** (HEEL), which was an ER retention motif found in PDIs of other species. Although *CsPDI* did not have the ER-retention signal, it was located, at least in part, in the ER and was supposed to play a critical role in the secretory pathway, just like in *Leishmania* (Padilla et al. 2003). Moreover, the identification of *CsPDI* as a component of *CsESPs* in our study will help corroborate our speculation.

As a multifunctional protein, another role of PDI was in catalyzing oxidation of disulfides of nascent polypeptides as it was usually abundantly expressed in the lumen of ER. However, in *N. caninum* (Naguleswaran et al. 2005), *T. gondii* (Meek et al. 2002a, b), and *Giardia* (Gillin et al. 1990), PDIs were found to be expressed on the surface of parasites. In *S. mansoni*, PDI was identified in the gastrodermis and protonephridia (Finken et al. 1994). In addition, PDI of *F. hepatica* has been reported as a component of *ESPs* to protect the parasite in oxidative stress environment (Salazar-Calderon et al. 2003). The *ESPs* of parasites often contained antioxidants which were shed from tegument, and proteases which were secreted from intestine (Berasain et al. 1997; Robinson et al. 2009). In *C. sinensis*, the *CsPDI* was located in the wall of intestine, which might help to explain why *CsPDI* was present in the *CsESPs*. Moreover, the specific localization of *CsPDI* was observed in the lumen of bile duct of the cat infected with *C. sinensis*, which might serve as another evidence of its role as a component of *CsESPs*.

The PDI of *Giardia lamblia* expressed on the surface was reported to play a role in protecting this protozoan in oxidative stress environment, which was considered to be one of mechanisms for the parasite surviving (Gillin et al. 1990). PDIs were expressed on the surface of *N. caninum* tachyzoites and *T. gondii* tachyzoites to functionally influence adhesive/invasive capacities of the tachyzoites (Lekutis et al. 2001; Naguleswaran et al. 2005). Recently, it has been shown that PDI was abundantly present in *Plasmodium micronema* and essential for parasite invasion (Lal et al. 2009). Therefore, further investigations are required to demonstrate whether *CsPDI* plays an important role in *C. sinensis* invasion and survival.

Immunohistochemical assay of *CsPDI* showed that it was localized in the vitellarium of adult worm and metacercaria. The vitellarium was a nutritive organ during sexual development, which was of great significance for

the growth of *C. sinensis*. Study in *S. mansoni* showed that PDI may take part in the nutrition supply to the germ cells, gastrodermis of gut, wall cells of protonephridia, and sustentacular cells of testes (Finken et al. 1994). It suggested that *CsPDI* may also be involved in *C. sinensis* growth and development.

Quantitative real-time PCR analysis revealed that the transcription level of *CsPDI* in the adult stage was lower than that in metacercaria and egg. It was possibly due to the fact that a large amount of *CsPDI* was lost as a component of *CsESPs* in the procedure of collecting adult worms. In this case, *CsPDI* was reduced in the total RNA of adult worms. On the other hand, the expression level of *CsPDI* mRNA in metacercaria presented the highest in three stages. It supposed that the protein played an important role in the growth and development of metacercaria, especially in the parasite invasion.

In this study, *CsPDI* was cloned, expressed, and purified using *E. coli* BL21 expression system. *CsPDI* was characterized as a novel molecule from *CsESPs*, and the protein may be of great importance in the parasite invasion and survival. Our studies will be the cornerstone of future researches on biological characterization, the role in host–parasite interplay, and the potential value as a therapy target in *C. sinensis* infection.

Acknowledgements This work was supported by the National Basic Research Program (973 program, 2010CB530000), “Eleventh Five-year Plan” for Science & Technology Research of China (2008ZX1004-011) and the National Natural Science Foundation of China (81101270).

References

- Berasain P, Goni F, McGonigle S, Dowd A, Dalton JP, Frangione B, Carmona C (1997) Proteinases secreted by *Fasciola hepatica* degrade extracellular matrix and basement membrane components. *J Parasitol* 83(1):1–5
- Byrne LJ, Sidhu A, Wallis AK, Ruddock LW, Freedman RB, Howard MJ, Williamson RA (2009) Mapping of the ligand-binding site on the b' domain of human PDI: interaction with peptide ligands and the x-linker region. *Biochem J* 423(2):209–217
- Chen W, Wang X, Deng C, Lv X, Fan Y, Men J, Liang C, Yu X (2011) Molecular cloning and characterization of a novel ras-related protein (rap2) from *Clonorchis sinensis*. *Parasitol Res* 108(4):1021–1026
- Finken M, Sobek A, Symmons P, Kunz W (1994) Characterization of the complete protein disulfide isomerase gene of *Schistosoma mansoni* and identification of the tissues of its expression. *Mol Biochem Parasitol* 64(1):135–144
- Freedman RB, Hirst TR, Tuite MF (1994) Protein disulphide isomerase: building bridges in protein folding. *Trends Biochem Sci* 19(8):331–336
- Freedman RB, Klappa P, Ruddock LW (2002) Protein disulfide isomerases exploit synergy between catalytic and specific binding domains. *EMBO Rep* 3(2):136–140
- Fried B, Abruzzi A (2010) Food-borne trematode infections of humans in the United States of America. *Parasitol Res* 106(6):1263–1280

- Fried B, Reddy A, Mayer D (2011) Helminths in human carcinogenesis. *Cancer Lett* 305(2):239–249
- Gilbert HF (1998) Protein disulfide isomerase. *Methods Enzymol* 290:26–50
- Gillin FD, Hagblom P, Harwood J, Aley SB, Reiner DS, McCaffery M, So M, Guiney DG (1990) Isolation and expression of the gene for a major surface protein of *Giardia lamblia*. *Proc Natl Acad Sci USA* 87(12):4463–4467
- Hong BX, Soong L (2008) Identification and enzymatic activities of four protein disulfide isomerase (PDI) isoforms of *Leishmania amazonensis*. *Parasitol Res* 102(3):437–446
- Hu F, Yu X, Ma C, Zhou H, Zhou Z, Li Y, Lu F, Xu J, Wu Z, Hu X (2007) *Clonorchis sinensis*: expression, characterization, immunolocalization and serological reactivity of one excretory/secretory antigen-LPAP homologue. *Exp Parasitol* 117(2):157–164
- Hu F, Hu X, Ma C, Zhao J, Xu J, Yu X (2009) Molecular characterization of a novel *Clonorchis sinensis* secretory phospholipase A (2) and investigation of its potential contribution to hepatic fibrosis. *Mol Biochem Parasitol* 167(2):127–134
- Kozlov G, Maattanen P, Thomas DY, Gehring K (2010) A structural overview of the PDI family of proteins. *FEBS J* 277(19):3924–3936
- Lal K, Prieto JH, Bromley E, Sanderson SJ, Yates JR, Wastling JM, Tomley FM, Sinden RE (2009) Characterisation of *Plasmodium* invasive organelles; an ookinete microneme proteome. *Proteomics* 9(5):1142–1151
- Lekutis C, Ferguson DJ, Grigg ME, Camps M, Boothroyd JC (2001) Surface antigens of *Toxoplasma gondii*: variations on a theme. *Int J Parasitol* 31(12):1285–1292
- Liao M, Ma L, Bannai H, Lee EG, Xie Z, Tang X, Zhang H, Xuan X, Fujisaki K (2006) Identification of a protein disulfide isomerase of *Neospora caninum* in excretory-secretory products and its IgA binding and enzymatic activities. *Vet Parasitol* 139(1–3):47–56
- Lun ZR, Gasser RB, Lai DH, Li AX, Zhu XQ, Yu XB, Fang YY (2005) Clonorchiasis: a key food borne zoonosis in China. *Lancet Infect Dis* 5(1):31–41
- Ma C, Hu X, Hu F, Li Y, Chen X, Zhou Z, Lu F, Xu J, Wu Z, Yu X (2007) Molecular characterization and serodiagnosis analysis of a novel lysophospholipase from *Clonorchis sinensis*. *Parasitol Res* 101(2):419–425
- Meek B, Back JW, Klaren VN, Speijer D, Peek R (2002a) Conserved regions of protein disulfide isomerase are targeted by natural IgA antibodies in humans. *Int Immunol* 14(11):1291–1301
- Meek B, Back JW, Klaren VN, Speijer D, Peek R (2002b) Protein disulfide isomerase of *Toxoplasma gondii* is targeted by mucosal IgA antibodies in humans. *FEBS Lett* 522(1–3):104–108
- Naguleswaran A, Alaeddine F, Guionaud C, Vonlaufen N, Sonda S, Jenoe P, Mevissen M, Hemphill A (2005) *Neospora caninum* protein disulfide isomerase is involved in tachyzoite-host cell interaction. *Int J Parasitol* 35(13):1459–1472
- Padilla A, Noiva R, Lee N, Mohan KV, Nakhasi HL, Debrabant A (2003) An atypical protein disulfide isomerase from the protozoan parasite *Leishmania* containing a single thioredoxin-like domain. *J Biol Chem* 278(3):1872–1878
- Pak JH, Moon JH, Hwang SJ, Cho SH, Seo SB, Kim TS (2009) Proteomic analysis of differentially expressed proteins in human cholangiocarcinoma cells treated with *Clonorchis sinensis* excretory-secretory products. *J Cell Biochem* 108(6):1376–1388
- Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29(9):e45
- Robinson CG, Roy CR (2006) Attachment and fusion of endoplasmic reticulum with vacuoles containing *Legionella pneumophila*. *Cell Microbiol* 8(5):793–805
- Robinson MW, Menon R, Donnelly SM, Dalton JP, Ranganathan S (2009) An integrated transcriptomics and proteomics analysis of the secretome of the helminth pathogen *Fasciola hepatica*: proteins associated with invasion and infection of the mammalian host. *Mol Cell Proteomics* 8(8):1891–1907
- Rubotham J, Woods K, Garcia-Salcedo JA, Pays E, Nolan DP (2005) Characterization of two protein disulfide isomerases from the endocytic pathway of bloodstream forms of *Trypanosoma brucei*. *J Biol Chem* 280(11):10410–10418
- Salazar-Calderon M, Martin-Alonso JM, Castro AM, Parra F (2003) Cloning, heterologous expression in *Escherichia coli* and characterization of a protein disulfide isomerase from *Fasciola hepatica*. *Mol Biochem Parasitol* 126(1):15–23
- Sripa B, Kaewkes S, Intapan PM, Maleewong W, Brindley PJ (2010) Food-borne trematodiasis in Southeast Asia epidemiology, pathology, clinical manifestation and control. *Adv Parasitol* 72:305–350
- Wilkinson B, Gilbert HF (2004) Protein disulfide isomerase. *Biochim Biophys Acta* 1699(1–2):35–44
- Yoo WG, Kim TI, Li S, Kwon OS, Cho PY, Kim TS, Kim K, Hong SJ (2009) Reference genes for quantitative analysis on *Clonorchis sinensis* gene expression by real-time PCR. *Parasitol Res* 104(2):321–328